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Social discrimination by quantitative assessment of immunogenetic similarity

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Genes of the major histocompatibility complex (MHC) that underlie the adaptive immune system may allow vertebrates to recognize their kin. True kin-recognition genes should produce signalling products to which organisms can respond. Allelic variation in the peptide-binding region (PBR) of MHC molecules determines the pool of peptides that can be presented to trigger an immune response. To examine whether these MHC peptides also might underlie assessments of genetic similarity, we tested whether *Xenopus laevis* tadpoles socially discriminate between pairs of siblings with which they differed in PBR amino acid sequences. We found that tadpoles (four sibships, $n = 854$) associated preferentially with siblings with which they were more similar in PBR amino acid sequence. Moreover, the strength of their preference for a conspecific was directly proportional to the sequence similarity between them. Discrimination was graded, and correlated more closely with functional sequence differences encoded by MHC class I and class II alleles than with numbers of shared haplotypes. Our results thus suggest that haplotype analyses may fail to reveal fine-scale behavioural responses to divergence in functionally expressed sequences. We conclude that MHC–PBR gene products mediate quantitative social assessment of immunogenetic similarity that may facilitate kin recognition in vertebrates.

Keywords: immunogenetics; kin recognition; major histocompatibility complex; MHC peptides; recognition alleles; sequence divergence

1. INTRODUCTION

Recognition mechanisms enable individuals to maintain their organismic integrity in the face of parasites, pathogens and competitors that might exploit them [1]. Beyond that, inclusive fitness should be maximized by recognition of genetic similarity [2], possibly facilitated by special kin-recognition genes [3,4]. Some behaviours directly covary with genetic relatedness [5], and putative kin-recognition genes have been identified [6–11]. If such genes function to enable organisms to recognize kin, we expect to find that social behaviours, where discrimination is adaptive, vary in direct response to the signalling products of these genes [12]. Here, we demonstrate that major histocompatibility complex (MHC)-based social preferences are mediated by signals that directly correlate with amino acid sequence similarity in the peptide-binding region (PBR) of MHC molecules.

Susceptibility to pathogens varies by individuals' MHC types [13–19]. MHC–PBR differences determine the binding affinity of MHC molecules to self-peptides and pathogen epitopes [20]. The pool of peptide ligands cleaved by MHC molecules reflects structural properties of the peptide-binding groove that are determined by the PBR amino acid sequence. Discrimination thresholds based on MHC–PBR amino acid sequence similarity have been documented in mammals [21], birds [22] and fishes [23–26]. This suggests that peptides restricted by the peptide-binding groove of MHC molecules directly or indirectly generate social recognition signals. Indeed, specific subsets of sensory neurons in the vomeronasal organ [27,28] and main olfactory epithelium [29] may detect and discern MHC genotype-specific pools of these peptides.

As highlighted in recent reviews [30–33], recognition systems that are encoded by special kin-recognition genes may not be evolutionarily stable. Recognition systems based on single genetic markers may lead individuals to falsely recognize non-kin with which they share alleles and to fail to recognize kin that bear different alleles [2,32,33]. Moreover, single-locus recognition systems should favour cooperation among conspecifics bearing common alleles, thereby limiting the diversifying selection required to maintain variation in markers [30,34]. Conversely, rare markers may become associated with higher levels of altruism in populations with low genetic recombination and dispersal rates [3,30,31]. However, the composition of an individual's pool of MHC-peptide ligands is shaped not only by its MHC type, but also by variation elsewhere in its genome [35]. Therefore, MHC-based recognition systems should be stable if the mediating social signals are composed of diverse MHC-peptide ligands, influenced by both MHC-type and genome-wide variation. In populations with low background genetic variation, such recognition systems should be sensitive in a quantitative manner to amino acid sequence variation in the PBR of MHC molecules.

Kin association is common in amphibian larvae [36–39] and may be based on recognition of MHC

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Table 1. Primer details.

haplotype	locus	primer direction	primer sequence	amplicon length (bp)	primer concentrations (pmol)	
					haplotype specific	control
f	class I-α1	sense	GTCTCAGATCGAGCCTTTGG	106	16.5	3.5
		antisense	TTGCAGGTTCATCTCTACCAGT		16.5	
g	class I-α1	sense	GTCTCAGATCGAGCCTTTGG	178	12.5	1.0
		antisense	GCTCTGATCCCTTGGCAAT		20.0	
j	class I-α1	sense	GTCTCAGATCGAACCTTTGG	178	15.0	0.8
		antisense	CCTCTTCTCCTTTCGCTTT		30.0	
r	class I-α1	sense	AGATAGAGCATTTGGGCTGC	134	21.2	2.5
		antisense	ATTCAGGTCCTGCTTTGTCC		21.2	
control	class I-α3	sense	TCACCCTCATGTAAGAATTTCAGA	236	n.a.	n.a.
		antisense	GCTCCACATGACAGGCATAA		n.a.	

types [39]. However, frog tadpoles discriminate even among their siblings, specifically associating with others with which they share MHC haplotypes in preference to those that bear different haplotypes [11]. In *Xenopus laevis*, a model organism for studying vertebrate immunology [40], we tested whether social discrimination varies proportionally to quantifiable signal differences as determined by amino acid sequence similarity in the MHC–PBR. If recognition is based on assessment of PBR sequence differences, we expected that association preferences would become stronger as sequence divergence increased between subjects and each of two simultaneously presented stimulus groups.

2. MATERIAL AND METHODS

(a) *Subjects*

We bred *X. laevis* from stock with known sequences for MHC class I and class II alleles. The haplotypes of linked MHC class I and II loci are defined as *f*, *g*, *j* and *r* (GenBank: class Ia accession numbers AF185579, AF185580, AF185582 and AF185586; class II accession numbers AF454374–AF454382) [41,42]. These strains originated from the Basel Institute for Immunology and had been bred for several generations in our laboratory.

We crossed pairs of MHC-heterozygous frogs that shared haplotypes (*rj* × *rj*, *rg* × *rg*, *fg* × *fg* and *fr* × *fr*), thereby producing clutches consisting of mixtures of homozygous and heterozygous full siblings (e.g. the *rj* × *rj* cross produced *rr*, *rj* and *jj* progeny). We reared tadpoles with their siblings in groups of 200 within 40 l tanks for two to three weeks and fed them by maintaining a suspension of finely ground nettle. We determined the MHC haplotypes of all stimulus and subject tadpoles by the polymerase chain reaction (PCR) using sequence-specific primers [11] from tail tip tissue before behavioural tests. After taking tail clips, we isolated tadpoles in 1 l polypropylene cups for one to four weeks, during which time tadpoles' tails fully regenerated, and we then tested them. At the time of testing, tadpoles' toes had not yet begun to differentiate (stage 54) [43].

(b) *Sequence-specific priming PCR major histocompatibility complex genotyping*

We extracted genomic DNA from tail tips using PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA). We MHC-typed tadpoles by PCR using sequence-specific primers for each of the four haplotypes (*f*, *g*, *j* and *r*; table 1), including a positive control that amplifies a conserved region of the MHC in each reaction to prevent failed reactions from being scored as negative.

Sequences were amplified on 96-well PCR plates (Axygen Scientific, PCR-96-C) in 12.5 µl PCRs, each containing 30–80 ηg of template DNA, PCR buffer (63.6 mM KCl, 127.2 mM Tris–HCl (pH 8.3), 1.9 mM $MgCl_2$), 180 µM dNTP (Invitrogen) and 0.2 unit *Taq* polymerase (Roche Diagnostics). Primer concentrations varied depending on the haplotype being assessed (table 1). The conditions for touchdown PCR in a thermocycler (Mastercycler Gradient; Eppendorf, Hamburg, Germany) were as follows: denaturation for 90 s at 94°C, followed by five cycles of denaturation for 30 s at 94°C, annealing for 45 s at 70°C and primer extension for 30 s at 72°C, followed by 20 cycles of denaturation for 30 s at 94°C, annealing for 50 s at 65°C and primer extension for 45 s at 72°C, followed by five cycles of denaturation for 30 s at 94°C, annealing for 1 min at 56°C and primer extension for 2 min at 72°C. We electrophoresed PCR products next to known positives and negatives for 40 min at 70 V in horizontal 2 per cent agarose gels. Gels were visualized by ethidium bromide fluorescence.

(c) *Association preference tests*

We simultaneously exposed subjects to two stimulus groups of 10 of their size- and stage-matched siblings on either side of a testing apparatus, separated by mesh net enclosures. Subjects shared different numbers (0, 1 or 2) of MHC haplotypes with each of the stimulus groups. We measured times spent by subjects associating with each of the groups.

Tests were conducted in polypropylene tanks (210 × 140 × 45 mm), with removable grey PVC-coated fibreglass (0.028 cm diameter) mesh (7.1 × 5.5 threads cm^{-1}) nets (43 × 140 × 45 mm) at each end, filled with 1.2 l of filtered deep-aquifer water at 21°C. A line drawn along the centre of each tank demarcated the two halves of the test arena (124 × 140 × 45 mm). Lighting was diffuse, achieved by reflecting two 100 W incandescent lamps off the ceiling of the test room.

We introduced test subjects by perforated spoon (to limit water transfer) into the centre of the apparatus. We allowed tadpoles to acclimate for 5 min and then tested them for

40 min. To eliminate any side bias, we tested each subject twice, reversing the stimulus groups after a water change. Consequently, each tadpole was tested for a total of 80 min. Tadpole association tests were recorded using a CCTV camera (Panasonic WV-BP330/G) with an adjustable focal lens (Panasonic WV-LZF61/2) positioned 1 m above the testing apparatus and a time-lapse (one-fifth speed) VHS recorder (Panasonic AG-TL350). We tracked movements of subjects from videotape using EthoVision v. 3.0 (Noldus Information Technology, Wageningen, The Netherlands). Time periods spent on either side of the centre line were computed for each subject.

We tested all possible association preferences among siblings: whether (i) MHC-homozygous subjects preferred their siblings with which they shared both MHC haplotypes to those with which they shared no MHC haplotypes (2 versus 0, $n = 262$); (ii) MHC-homozygous subjects preferred siblings sharing both MHC haplotypes to those with which they shared only one MHC haplotype (2 versus 1, $n = 187$); and (iii) MHC-homozygous subjects preferred siblings with which they shared only one MHC haplotype to those with which they shared no MHC haplotypes (1 versus 0, $n = 199$). We also tested MHC-heterozygous subjects to determine whether they discriminated between siblings with which they shared one or both MHC haplotypes (heterozygotes, $n = 206$). Sample sizes varied among genotypes within families dependent on the availability of genotyped progeny of appropriate developmental stage (table 2). The behavioural data on which analyses were based have been deposited in the Dryad data repository (doi:10.5061/dryad.2204v).

For each choice test type, we evaluated the overall effect of MHC similarity on subjects' association preferences by nested analysis of variance using type III sums of squares [44]. To distinguish between association preferences of the haplotypes within families while maintaining statistical independence, we compared alternate subjects of each haplotype for the time spent associating with siblings sharing more MHC haplotypes with that spent associating with siblings sharing fewer MHC haplotypes. The effects of MHC similarity, genotype nested within MHC similarity, and family nested within genotype and MHC similarity were included as factors in each analysis. Data met assumptions of normality. Analyses were conducted with JMP v. 8.0.2 (SAS Institute, 2009). All statistical inferences were drawn on two-tailed distributions with $\alpha = 0.05$.

For each choice test, in each of the four families, we calculated the percentage of amino acids shared at the MHC class I (α1 and α2 domain exons) and MHC class II PBRs (α1 and α2 domain exons of the DAA and DBA loci) between test subjects and each of the two stimulus groups from published sequences [41,42]. We then calculated the 'stimulus differential' between the subject and the two stimulus groups by subtracting the per cent amino acid similarity of the less MHC-similar stimulus group from that of the more MHC-similar stimulus group. The stimulus differential is a function of the type of choice test (1 versus 0; 2 versus 1; and 2 versus 0 shared haplotypes, heterozygotes) and of the sequence differences between the haplotypes.

We correlated subjects' time preference for the more MHC-similar stimulus group with the corresponding stimulus differential scores, computed separately for MHC class I and II PBR amino acid sequences. We did not include the MHC class II DCA locus in the analysis as it has only been partially sequenced for the *f* haplotype and is expressed in very low amounts, if at all [42]. Spearman's rank correlations were conducted with JMP v. 8.0.2.

Table 2. Tadpole association preferences by subject group.

number of shared haplotypes	subject MHC type	parental cross	stimulus MHC types	*n*
2 versus 0	*ff*	*fg* × *fg*	*ff* versus *gg*	37
	ff	*fr* × *fr*	*ff* versus *rr*	41
	gg	*fg* × *fg*	*gg* versus *ff*	41
	gg	*rg* × *rg*	*gg* versus *rr*	31
	jj	*rj* × *rj*	*jj* versus *rr*	16
	rr	*rj* × *rj*	*rr* versus *jj*	19
	rr	*rg* × *rg*	*rr* versus *gg*	41
	rr	*fr* × *fr*	*ff* versus *rr*	36
2 versus 1	*ff*	*fg* × *fg*	*ff* versus *fg*	23
	ff	*fr* × *fr*	*ff* versus *fr*	40
	gg	*fg* × *fg*	*gg* versus *fg*	23
	gg	*rg* × *rg*	*gg* versus *gr*	30
	jj	*rj* × *rj*	*jj* versus *jr*	10
	rr	*rj* × *rj*	*rr* versus *jr*	9
	rr	*rg* × *rg*	*rr* versus *gr*	25
	rr	*fr* × *fr*	*rr* versus *fr*	27
1 versus 0	*ff*	*fg* × *fg*	*fg* versus gg	20
	ff	*fr* × *fr*	*fr* versus *rr*	40
	gg	*fg* × *fg*	*fg* versus *ff*	29
	gg	*rg* × *rg*	*gr* versus *rr*	34
	jj	*rj* × *rj*	*jr* versus *rr*	16
	rr	*rj* × *rj*	*jr* versus *jj*	8
	rr	*rg* × *rg*	*gr* versus *gg*	20
	rr	*fr* × *fr*	*fr* versus *ff*	32
heterozygotes	*fg*	*fg* × *fg*	*fg* versus *ff*	26
	fg	*fg* × *fg*	*fg* versus *gg*	25
	rg	*rg* × *rg*	*rg* versus *rr*	26
	rg	*rg* × *rg*	*rg* versus *gg*	23
	rj	*rj* × *rj*	*rj* versus *jj*	19
	rj	*rj* × *rj*	*rj* versus *rr*	28
	fr	*fr* × *fr*	*fr* versus *ff*	31
	fr	*fr* × *fr*	*fr* versus *rr*	28

3. RESULTS

Tadpoles associated preferentially with the stimulus group with which they shared more MHC haplotypes. The magnitude of the preference increased in direct proportion to the stimulus differential score computed for each test (four sibships, $n = 854$; figure 1). We obtained similar results when considering MHC class I ($r_S = 0.14$, $p < 0.0001$) and class II ($r_S = 0.14$, $p < 0.0001$) PBR amino acid sequences. Subjects showed stronger preferences for their own haplotype when given a choice between sibling groups with more divergent PBR sequences.

Analysis of association preferences by PBR sequence divergence reveals fine-grained recognition abilities that do not emerge as clearly from haplotype analyses. Tadpoles preferred siblings with which they shared both MHC haplotypes to those with which they shared one (2 versus 1; $F_{1,171} = 5.39$, $p = 0.021$) or no (2 versus 0; $F_{1,246} = 21.39$, $p < 0.001$) MHC haplotypes (figure 2). However, we found no significant preferences for siblings with which subjects shared only one MHC haplotype to those with which they shared none (1 versus 0; $F_{1,183} = 0.25$, $p = 0.62$). MHC heterozygotes also showed no preference between stimulus groups that bore different

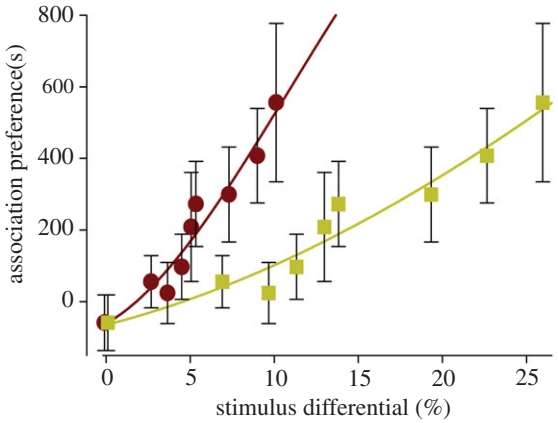

Figure 1. Association preference of subjects for the more immunogenetically similar stimulus group as a function of the MHC 'stimulus differential' between the two stimulus groups based on MHC class I (circles) and MHC class II PBR (squares) amino acid sequence similarity. The stimulus differential between each subject and the two stimulus groups was determined by subtracting the per cent amino acid similarity of the less MHC-similar stimulus group from that of the more MHC-similar stimulus group. Means ± s.e.m. are shown.

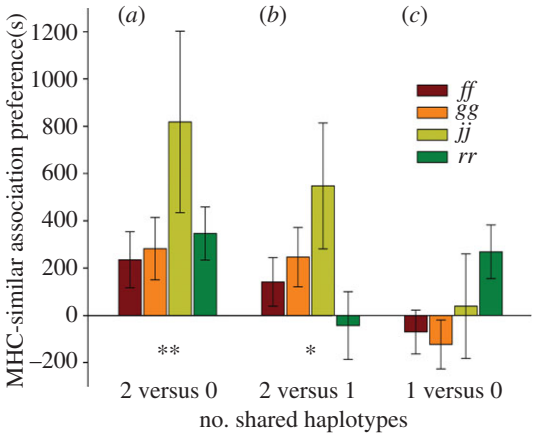

Figure 2. Mean MHC-similarity preferences of MHC-homozygous subjects (four genotypes: *ff*, *gg*, *jj* and *rr*) among stimulus groups with different numbers of shared MHC haplotypes (2 versus 0 shared haplotypes; 2 versus 1 shared haplotypes; 1 versus 0 shared haplotypes). (*a*) Subjects spent more time associating with siblings sharing both MHC haplotypes than with siblings sharing no MHC haplotypes (2 versus 0). (*b*) Subjects spent more time associating with siblings sharing both MHC haplotypes than with siblings sharing only one MHC haplotype (2 versus 1). (*c*) Subjects did not differ in time associating with siblings sharing one or no MHC haplotypes (1 versus 0). Means ± s.e.m. are shown. $*p < 0.05$, $**p < 0.001$ (two-tailed).

combinations of shared MHC haplotypes ($F_{1,190} = 0.46$, $p = 0.50$). See table 3 for full ANOVA results.

4. DISCUSSION

Xenopus laevis tadpoles socially discriminate among their siblings based on quantitative assessment of their MHC–PBR amino acid sequence differences. While subjects' discrimination among siblings varied by numbers of shared MHC haplotypes, overall MHC-assortative preferences, spanning all haplotypes, correlated more closely with functional sequence differences in the PBR of MHC molecules. Analyses of PBR sequence differences thus are more robust than haplotype analyses in explaining the behavioural discrimination that we observed. Rather than recognizing on the basis of shared MHC alleles, our results suggest that tadpoles assess differential 'MHC-signal' strength, determined by the PBR amino acid sequences encoded by MHC alleles.

Thus, even when they share equal proportions of other genes identical by descent, *X. laevis* tadpoles discriminate among siblings by effectively comparing functional properties of their own MHC–PBR with those of others. Association preferences correspond to individuals' allelic similarity, specifically in MHC class I and class II loci, dependent on shared ligand anchor residues (PBR amino acids) encoded by these genes. These results provide the strongest evidence yet that factors associated with the binding specificity of the peptide-binding groove of MHC molecules can elicit kin recognition. By testing only siblings, our experimental design controlled for recognition possibly based on products of other genes.

Differences in MHC-ligand binding efficiency can influence markers used in MHC-type recognition either by restricting different excreted peptide sequences [27,45] or by selecting the microbial biota associated with individuals [46–48]. The peptide-binding groove of MHC molecules generates a pool of 9-mer peptides cleaved from longer protein sequences. These 9-mers are individually distinctive [49] and may serve as markers of overall genetic relatedness. While further work is required to determine the mechanism by which MHC ligand peptides stimulate larval olfactory mucosa, our findings suggest that these small 9-mer subunits carry sufficient information for social discrimination of kin.

The MHC–PBR determined pool of peptides that serve as ligands for MHC molecules [20,49] contributes to individual odour profiles [50] that have been implicated in individual preferences of mice [27–29] and fish [45]. Pregnant mice are more likely to undergo pregnancy block if exposed to synthesized 9-mers based on disparate rather than familiar MHC class I peptide ligands [27]. Similarly, mate choice decisions of female sticklebacks can be predictably modified by adding different combinations of synthetic 9-mer peptides [45]. Sticklebacks discriminate cues of potential mates based on their diversity of MHC alleles across multiple MHC class II loci [51,52].

Unlike class II molecules, class I molecules have not been detected in *X. laevis* tadpoles at the developmental stages that we examined [53]. Nonetheless, because MHC class I mRNA transcripts have been detected in organs whose epithelial surfaces are in contact with the environment, such as lungs, gills and intestines [54], the class I locus is as likely as the class II loci to be involved in MHC-type discrimination. As MHC class I transcripts in tadpoles are limited mainly to tissues in contact with the external environment, excreted MHC peptides may be sufficient for the transmission of MHC-specific signals in an aqueous environment.

Our results demonstrate that behavioural responses can be elicited by quantitative evaluation of MHC–PBR amino acid sequence differences. Certainly, many types of cues aside from those related to the MHC influence social preferences [9,32,39] but discrimination based on those cues

Table 3. Analysis of variance for association preference tests.

source	d.f.	MS	*F*	*p*
2 versus 0 shared haplotypes				
MHC similarity	1	7 003 243	21.39	<0.001
genotype (MHC similarity)	6	229 638	0.70	0.65
sibship (genotype (MHC similarity))	8	23 550	0.072	1.00
residual error	246	327 341	—	—
2 versus 1 shared haplotypes				
MHC similarity	1	1 275 406	5.39	0.021
genotype(MHC similarity)	6	197 578	0.84	0.54
sibship (genotype (MHC similarity))	8	98 590	0.42	0.91
residual error	171	236 600	—	—
1 versus 0 shared haplotypes				
MHC similarity	1	42 438	0.25	0.62
genotype (MHC similarity)	6	285 413	1.65	0.14
sibship (genotype (MHC similarity))	8	76 975	0.44	0.89
residual error	183	173 011	—	—
heterozygotes				
MHC similarity	1	145 364	0.46	0.50
genotype (MHC similarity)	6	28 267	0.090	1.00
sibship (genotype (MHC similarity))	8	412 578	1.31	0.24
residual error	190	315 142	—	—

was not possible in this study. However, this study points to the possibility that MHC molecules also may facilitate recognition of genome-wide sequence differences that contribute to the composition of individuals' MHC-restricted peptide ligands.

That the same genetic sequence polymorphisms determine self/non-self recognition and social compatibility suggests a shared functional framework driving the evolution of MHC diversity [55,56]. Fine-scale quantitative assessment of MHC-similarity may permit the recognition not only of closely related individuals [33], but also of possible disease risks associated with immunogenetic compatibility [17,18]. Analyses based on fine-scale divergence in functionally expressed sequences can reveal genetic effects on important biological traits that simple examination of genotypes may fail to discern.

All protocols involving animals were approved by the Animal Ethics Committees of Lincoln University, Seoul National University and the University of Canterbury.

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